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# Trematode infection modulates cockles biochemical response to climate change

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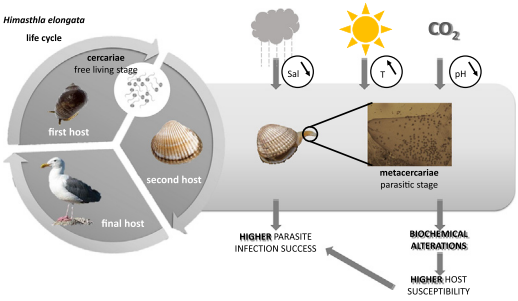
## HIGHLIGHTS

- Higher infection success with water salinity decrease, warming and acidification
- Under lower water salinity, parasite infection reduced cockle antioxidant defence.
- Under higher water temperature, parasite infection increased cockle cellular damage.
- Under higher $pCO_2$, parasite infection induced cockle metabolic depression.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Resulting mainly from atmospheric carbon dioxide ($CO_2$) build-up, seawater temperature rise is among the most important climate change related factors affecting costal marine ecosystems. Global warming will have implications on the water cycle, increasing the risk of heavy rainfalls and consequent freshwater input into the oceans but also increasing the frequency of extreme drought periods with consequent salinity increase. For Europe, by the end of the century, projections describe an increase of $CO_2$ concentration up to 1120 ppm (corresponding to 0.5 pH unit decrease), an increase in the water temperature up to 4 °C and a higher frequency of heavy precipitation. These changes are likely to impact many biotic interactions, including host–parasite relationships which are particularly dependent on abiotic conditions. In the present study, we tested the hypothesis that the edible cockle, *Cerastoderma edule*, exposed to different salinity, temperature and pH levels as proxy for climate change, modify the infection success of the trematode parasite *Himasthla elongata*, with consequences to cockles biochemical performance. The results showed that the cercariae infection success increased with acidification but higher biochemical alterations were observed in infected cockles exposed to all abiotic experimental stressful conditions tested. The present study suggested that changes forecasted by many models may promote the proliferation of the parasites infective stages in many ecosystems leading to enhanced transmission, especially on temperate regions, that will influence the geographical distribution of some diseases and, probably, the survival capacity of infected bivalves.



## 1. Introduction

It is of prime relevance to understand the consequences imposed by occurring and predicted climate change (IPCC, 2014) on marine species interactions such as host-parasite systems. Climate-related changes,

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namely sea level rise, increasing storm frequency, temperature rise and ocean acidification are expected to have negative consequences particularly for the biota of intertidal and shallow marine areas (Ringwood and Keppler, 2002). These consequences include biodiversity and habitat reduction, shifts of species distributional patterns, and/or even ecosystem loss (Brierley and Kingsford, 2009; Cheung et al., 2009; Doney et al., 2012; Sarà et al., 2014). Effects on one species can drive changes up to the population or community levels. For example, effects at the individual reproduction level can drive changes on the population recruitment which in turn can influence the interactions among populations at the community level, such as predation and competition (Harley et al., 2006). Therefore, predicted changes are likely to impact many biotic interactions including host–parasite relationships which are particularly dependent on abiotic conditions (de Montaudouin et al., 2016). In fact, the negative effects of infectious diseases may become more severe in a global climate change scenario (Harvell et al., 2002).

Parasitic fauna represents 40% of total known eukaryotic species (Dobson et al., 2008) and can influence the composition and structure of natural animal communities being important elements of worldwide ecosystems (Poulin, 1999). However, studies converging parasitology and climate change are still scarce and focused mainly on cercariae emergence and survival (MacLeod, 2017). Increasing of river flows (Robins et al., 2016) and shifts on the rainfalls frequency (Feyen and Dankers, 2009; Robins et al., 2016) are among the environmental factors derived from climate change which can impact the aquatic organisms. These changes will have a significant impact on the salinity of the aquatic systems, especially noticed in lagoons and estuaries (Schmitt, 2008). Salinity plays an important role in defining structural and functional characteristics of aquatic biota (Telesh and Khlebovich, 2010; Fazio et al., 2013) and has been recognised as an important driver for parasitism and disease dynamics (Messick et al., 1999; Coffey et al., 2012).

According to recent studies (IPCC, 2014), climate change will also include the increase of earth surface temperature up to 4 °C by the end of the 21st century. Temperature is among the most pervasive and important physical factors in the environment of an organism and can have implications from molecular to biogeographical levels (Somero, 2011). Temperature effects on parasites are well described, demonstrating that an increase in temperature induces higher cercariae emergence until an optimum (Mouritsen, 2002; Thieltges and Rick, 2006; Studer et al., 2010; Koprivnikar et al., 2014) and higher cercariae infectivity (Thieltges and Rick, 2006; Studer et al., 2010) but lower cercariae survival (Mouritsen, 2002; Thieltges and Rick, 2006; Studer et al., 2010).

Oceanic partial pressure of carbon dioxide ($pCO_2$) has been increasing. In 2011 ocean pH had already decreased by 0.1 pH unit since the beginning of the industrial era and is predicted to decrease further by 0.3 to 0.5 pH unit (Caldeira and Wickett, 2003). Beyond implications of low pH on shell production due to a decrease in calcium carbonate availability (Raven et al., 2005), the most important effect of acidification is on the increased organisms metabolic demand for the acid-base regulation which can reduce the amount of energy available for other activities such as respiration, growth and reproduction (Kroeker et al., 2010). The specific effects of ocean acidification on host-parasite interactions are almost unknown although some evidences suggest significantly higher infection success in the environment subjected to reduced pH levels (Koprivnikar et al., 2010; Harland et al., 2016).

Despite increasing efforts on parasitological studies over the last decades (Poulin et al., 2016), parasites still play a discrete role in marine ecosystems and little is known about how parasites are influenced by environmental changes (Koprivnikar et al., 2010; Studer and Poulin, 2013). Therefore, in the present study, we used the edible cockle, *Cerastoderma edule*, as a host model and the trematode parasite, *Himasthla elongata* as a parasite model, to test the hypothesis that host exposure to different salinity, temperature and pH levels, as proxy for climate change, can modify the infection success of the parasite. Besides, this work aimed to investigate the interactive effects of each abiotic variable tested (salinity, temperature and pH) and trematode infection on the host biochemical performance. Accordingly, a series of laboratory experiments were conducted by exposing cockles as second intermediate host of *H. elongata* cercariae, to the three distinct experimental conditions.

## 2. Material and methods

### 2.1. Hosts and parasites

The parasite species used was *Himasthla elongata*, a marine trematode, i.e. the most dominant clade of macroparasites in coastal waters (Lauckner, 1983). This parasite has a complex life cycle with three host species: i) a water bird as definitive host, where the adult parasitic stage develops and sexually reproduces; ii) the gastropod *Littorina littorea* as first intermediate host, where the sporocyst parasitic stage matures and the cercariae are formed and released (asexual multiplication). Usually, cercariae display a short lifespan (<48 h) and their functional ability to infect has a duration of <12 h (de Montaudouin et al., 2016); iii) next, *H. elongata* cercariae penetrate a cockle as second intermediate host and settle as metacercariae.

Potential first intermediate host *L. littorea* infected with *H. elongata* were collected from NIOZ harbour, Texel, the Netherlands (53° 00′ 32.1″N, 4° 47′ 36.5″E) in September 2017. Returning from the field, snails were screened for infections by incubating them in wells (6-well plate) with ~16 mL seawater (salinity = 35 ± 1) at 25 °C under constant illumination for 4 h. After, each well of each plate was observed at the stereomicroscope to check for the presence or absence of emitted cercariae in the water where the snails were immerged. Non-infected snails were returned to their natural habitat, while infected snails, a stock composed by six individuals (shell height ranged between 14 and 18 mm), were transported to Portugal at room temperature and dark conditions. When in the laboratory, snails were kept in the dark, in a 70 L aquarium filled with artificial seawater (salinity = 35 ± 1) constantly refreshed by a chiller to maintain 14 ± 1 °C and fed with fresh sea lettuce (*Ulva* sp.) ad libitum.

Potential second intermediate host *Cerastoderma edule* (edible cockle) was collected from the Mira channel, Ria de Aveiro coastal lagoon, Portugal (40° 38′ 31.7″N, 8° 44′ 10.9″W) in September 2017. After collection, cockles were transferred to the laboratory and acclimated for two weeks prior to exposure. The cockles were kept randomly distributed in two aquaria with constant filtration under controlled (CTL) conditions: salinity = 30 ± 1, temperature = 17 ± 0 °C, pH = 8.2 and photoperiod = 12:12 h (light/dark). Cockles were fed with a heterotrophic and phototrophic species mixture (Algamac Protein Plus®) at a concentration of 720 cells $\mu L^{-1}$ $day^{-1}$ adapted from Pronker et al. (2015). Cockles shell length ranged between 13 and 17 mm, i.e. young individuals, in order to limit natural trematode former infection.

### 2.2. Experimental design

In order to stimulate cercariae emergence from the infected snails, these organisms were individually transferred to a plate well (6-well plate) with ~16 mL artificial seawater (salinity = 35 ± 1) and exposed to constant illumination and, consequently, higher temperature (~25 °C) during a period that ranged between 4 and 6 h. Emitted cercariae were then individually collected with a pipette and separated into groups of twenty five for immediate cockles infestation.

After cockles acclimation period (see Section 2.1), three different experiments were carried out with sixty cockles (thirty cockles per treatment from which fifteen were infected) and 750 cercariae (Fig. 1). Experiments lasted for 144 h (96 h + 48 h) and were performed in three interspersed weeks to allow snails and parasites recovery and consequent maintenance of the cercariae production.

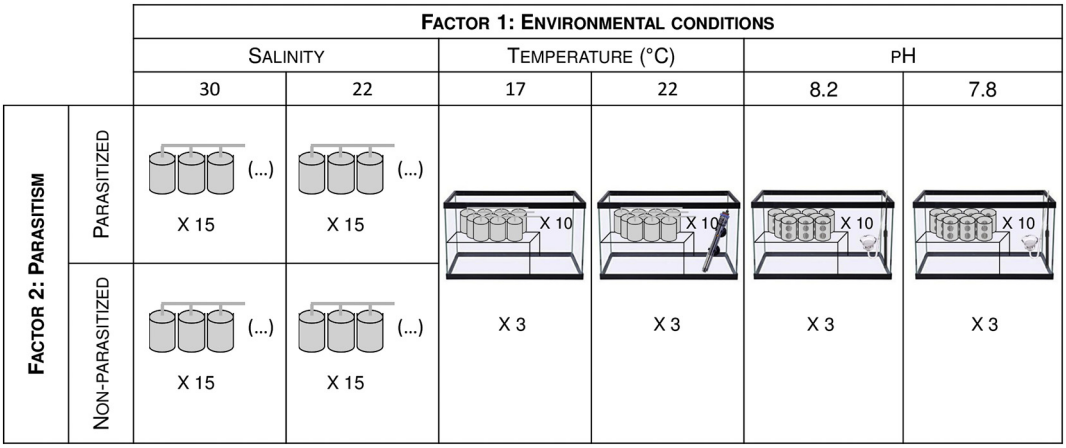


**Fig. 1.** Schematic representation of the experimental designs. Each experiment contained sixty replicates, thirty per each treatment level (two salinity, temperature and pH levels) and fifteen per each treatment level × infection condition (parasitized and not parasitized).

Each experiment consisted on two phases: (1) incubation period – during 96 h, cockles were exposed to the different treatments of salinity, temperature or pH (according to the experiment) with two levels per condition; (2) infection period – fifteen cockles per treatment (thirty cockles per experiment) were individually infested with *H. elongata* by dropping twenty-five cercariae from a microtube to the water of the cockles containers. During 48 h, cockles were simultaneously exposed to both factors: salinity and parasites, temperature and parasites or pH and parasites, according to the experiment (Fig. 1). All experiments were conducted with artificial seawater prepared at required salinity with reverse osmosis water and artificial sea salt (RedSea Salt®).

*2.2.1. Salinity experiment*

The levels tested were salinity 30 (salinity control, CTL), resembling conditions at the sampling area (Magalhães et al., 2018), and salinity 22, corresponding to a salinity decrease derived from an increase in the frequency and intensity of precipitation events predicted for Europe, especially in winter (IPCC, 2014). During the first week of the experimental period, to achieve testing salinity 22, the salinity of one group of cockles was lowered at a rate of 2 every other day. After this procedure, for each salinity level (2 levels), thirty cockles were individually placed in glass containers, filled with 50 mL seawater at the required salinity (30 or 22) and with constant aeration (Fig. 1). Per salinity level (2 levels) and after the incubation period (96 h of exposure at each salinity), fifteen cockles were individually parasitized (for 48 h) with twenty five cercariae per cockle. At the end of the experiment (144 h), nine cockles were dissected to measure infection success and six were properly tagged and conserved at −80 °C for biochemical analysis. Temperature and pH were maintained stable at control conditions (17.3 ± 0.3 °C and 8.2 ± 0.0, respectively) with 12:12 h (light/dark) photoperiod.

*2.2.2. Temperature experiment*

The levels tested were 17 °C (temperature control, resembling conditions at the sampling area (Magalhães et al., 2018), CTL) and 22 °C to test the effects of the predicted ocean warming (IPCC, 2014). For each temperature level, three aquaria were used, each one with ten cockles individually placed in glass containers, filled with 50 mL seawater and with constant aeration (Fig. 1). Increased temperature level was obtained by heating with a thermostat the aquaria water where cockles were placed (Fig. 1). When expected temperature was reached, cockles were maintained under experimental conditions (2 temperature levels) during 96 h and then five cockles per aquarium (a total of 15 cockles per treatment) were infected (for 48 h) with twenty five cercariae each. At the end of the experiment (144 h), cockles were dissected to measure infection success (nine individuals) or properly tagged and conserved at −80 °C for biochemical analysis (six individuals). Salinity and pH were maintained stable at control conditions (30 ± 1 and 8.2, respectively) with 12:12 h (light/dark) photoperiod.

*2.2.3. pH experiment*

The levels tested were 8.2 (pH control, based on the sampling area registered values, CTL) and 7.8, obtaining 403 ± 19 μatm of $pCO_2$ and 1112 ± 149 μatm of $pCO_2$ respectively (Table 1), an increase considered within $pCO_2$ predictions by the end of the twenty first century (Raven et al., 2005; IPCC, 2014). For each pH level, three aquaria were used with ten cockles each. In order to separate cockles (for later individual infection) but still maintain water circulation and pH conditions, cockles were isolated by pierced plastic containers covered by a 250 μm mesh net and filled with 50 mL seawater. Since *H. elongata* cercariae mean size is 400 × 180 μm (Krupenko and Dobrovolskij, 2015), this net avoided larvae escape. Acidification was obtained by directly diffusing $CO_2$ into aquaria and continuously monitored and controlled using a pH Stat system (Aquamedic®) (Fig. 1). When expected pH was reached, cockles were maintained under experimental conditions (2 pH levels) for 96 h, infected (for 48h) with twenty five cercariae each and at the end of the experiment (144 h) dissected to measure infection success (nine individuals) or properly tagged and conserved at −80 °C for biochemical analysis (six individuals). Salinity and temperature were maintained stable (30 ± 1 and 17.3 ± 0.3 °C, respectively) with 12:12 h (light/dark) photoperiod. Three water samples were collected from each aquaria at the experiment start (T0h), middle (T72h) and end (T144h) for further total alkalinity (TA) calculation using the Alkalinity calculator (USGS, 2012) after potentiometric titration (Gran, 1952). TA values and measured parameters (temperature, salinity and pH values, read at the time of water sampling) were plotted in $CO_2$Calc software (Robbins et al., 2010) and carbonate chemistry determined

**Table 1**
Mean ± standard deviation of carbonate system physicochemical parameters for each condition. Measured parameters: salinity, temperature and pH; determined parameters: Total alkalinity (TA), partial $CO_2$ pressure ($pCO_2$), bicarbonate ($HCO_3^-$), carbonate ion concentration ($CO_3^{2-}$) and saturation states of calcite (Ω Cal) and aragonite (Ω Ag). CTL: Control (field condition).

| | CTL (8.2) | CTL − 0.4 units (7.8) |
|---|---|---|
| Salinity | 30.0 ± 0.0 | 30.0 ± 0.0 |
| Temperature | 17.4 ± 0.1 | 17.2 ± 0.1 |
| pH | 8.18 ± 0.01 | 7.78 ± 0.04 |
| TA | 2291 ± 101 | 2241 ± 189 |
| $pCO_2$ (μatm) | 403 ± 19 | 1112 ± 149 |
| $HCO_3^-$ (μmol $kg^{-1}$) | 1906 ± 85 | 2074 ± 181 |
| $CO_3^{2-}$ (μmol $kg^{-1}$) | 158 ± 8 | 68 ± 7 |
| Ω Cal | 3.89 ± 0.20 | 1.68 ± 0.18 |
| Ω Ara | 2.48 ± 0.13 | 1.07 ± 0.11 |

using dissociation constants K1, K2 (Mehrbach et al., 1973; Dickson and Millero, 1987) and KHSO4 (Dickson, 1990). Mean values of each parameter were calculated considering all samples and collection periods.

### 2.3. Biochemical descriptors

Several studies showed that biochemical markers are useful tools to determine the impact of several stressors on marine bivalves (Faggio et al., 2016), including cockles (Freitas et al., 2012; Marques et al., 2016). In the present study, biochemical alterations induced by abiotic factors, by parasitism or by the interactive effects, were assessed measuring the energy reserves content namely protein (PROT) and glycogen (GLY); the activity of antioxidant enzymes namely superoxide dismutase (SOD), that converts superoxide anion ($O_2^-$) to hydrogen peroxide ($H_2O_2$), catalase (CAT) and glutathione peroxidase (GPx) both converting the SOD product ($H_2O_2$) to water; the activity of biotransformation enzymes namely glutathione S- transferases (GSTs) that catalyse the conjugation of the reduced form of glutathione to xenobiotics acting as cell detoxifier; the metabolic capacity through the electron transport system (ETS) activity and the level of cellular damage through the lipid peroxidation (LPO) quantification in organisms after exposure.

Cockles were pooled in groups of two specimens accounting for three replicates (corresponding to six cockles) per condition (2 salinity, temperature or pH levels × 2 infection conditions: parasitized (P) and non-parasitized (NP)). Each replicate was homogenised with liquid nitrogen and separated into 0.3 g of soft tissue subsamples in order to perform the extraction with three different buffers (1:2 w/v). Supernatant of the subsample extracted with phosphate buffer (50 mM $KH_2PO_4$ (pH 7.0), 1 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) PVP, 1 mM DTT) and centrifuged at 4 °C, 10,000*g* during 20 min was used to determine PROT and GLY concentrations, SOD, CAT, GPx and GSTs activities. Supernatant of the subsample extracted with 0.1 M Tris-HCl (pH 8.5), 15% (w/v) Poly Vinyl Pyrrolidone, 153 μM $MgSO_4$ and 0.2% (w/v) Triton X-100 buffer and centrifuged at 4 °C, 3000*g* during 20 min was used to determine ETS activity. Supernatant of the subsample extracted with 20% (w/v) trichloroacetic acid (TCA) and centrifuged at 4 °C, 10,000*g* during 20 min was used to determine LPO. All supernatants were used immediately after extraction or preserved at −20 °C for a short period of time.

Total PROT content was determined according to Robinson and Hogden (1940), following the Biuret method that uses Bovine serum albumin (BSA) as standard (0–40 mg $mL^{-1}$). After 10 min incubation at 30 °C the absorbance was read at 540 nm. The results were expressed in mg and used to calculate enzymes activity. The GLY content was determined by the phenol-sulphuric acid method (Dubois et al., 1956). Absorbance was measured at 492 nm and results were expressed in mg per grams (g) of fresh weight (FW).

The activity of SOD was measured using the method described by Beauchamp and Fridovich (1971). The standard curve was determined with SOD standards (0.25–60 U $mL^{-1}$) and the reaction was performed during 20 min in an orbital incubator set at room temperature. The enzyme activity was measured spectrophotometrically at 560 nm and expressed in enzyme unit (U) per mg of PROT. One U corresponds to a reduction of 50% of nitro blue tetrazolium (NBT). The activity of CAT was measured by the reaction of the enzyme with methanol in the presence of $H_2O_2$ (Johansson and Borg, 1988). The standard curve was determined using formaldehyde standards (0–150 mM) and the reaction was performed during 20 min in an orbital incubator set at room temperature. The formaldehyde formation in the presence of Purpald was spectrophotometrically measured at 540 nm and the enzymatic activity was expressed in U per mg of PROT. One U is defined as the amount of enzyme that generated the formation of 1.0 nmol formaldehyde per min. The activity of GPx was determined by the reaction of reduced glutathione, cumene hydroperoxide, glutathione reductase and NADPH (Paglia and Valentine, 1967). The NADPH oxidation is accompanied by a decrease in absorbance spectrophotometrically measured at 340 nm. The activity was calculated using $\varepsilon = 6.22 \times 10^{-3}\ M^{-1}\ cm^{-1}$ and expressed in U per mg of PROT. One U is defined as nmol of NADPH oxidized per min.

The activity of GSTs was determined using CDNB as substrate according to Habig et al. (1974) method. The increase in absorbance was spectrophotometrically measured at 340 nm. The activity was calculated using $\varepsilon = 9.60 \times 10^{3}\ M^{-1}\ cm^{-1}$ and expressed in U per mg of PROT. One U is defined as nmol of CDNB conjugate formed per min.

The ETS activity was determined by the amount of formazan formed after adding *p*-iodonitrotetrazolium (De Coen and Janssen, 1997), calculated using $\varepsilon = 15.9 \times 10^{3}\ M^{-1}\ cm^{-1}$ and expressed in nmol of formazan formed per min per g of FW.

Finally, LPO was measured by the quantification of thiobarbituric acid reactive substances (TBARS) (Buege and Aust, 1978). This methodology is based on the reaction of LPO by-products, namely malondialdehyde (MDA), with 2-thiobarbituric acid (TBA) forming TBARS. The amount of MDA was quantified spectrophotometrically and measured at a wavelength of 532 nm ($\varepsilon = 156 \times 10^{3}\ M^{-1}\ cm^{-1}$). Results were expressed as nmol of MDA equivalents per g FW.

### 2.4. Data analysis

For each experiment, two two-way ANOVAs were performed. The first used to verify cockle shell length similarity among treatments and the second used to test treatment (salinity, temperature and pH levels), infection condition (parasitized and non-parasitized) and the interaction between factors on the number of *H. elongata* metacercariae infecting cockles after exposure. Prior to analysis, homogeneity of variance was verified with Cochran test. Two-way ANOVAs were followed by post-hoc Dunnett test for comparison of means.

Due to a lack of homogeneity of variance, PROT, GLY, SOD, CAT, GPx, GSTs, ETS and LPO were separately submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in Primer v6) with a two factors design: abiotic treatment (salinity, temperature and pH) as factor 1 and infection condition (non-parasitized (NP) and parasitized (P)) as factor 2. PERMANOVA main test was performed to test the effect of treatment, infection condition and the interaction between these two factors on each biomarker. PERMANOVA main tests were considered significant when $p < 0.05$ and followed by PERMANOVA pair-wise tests. Pair-wise tests were used to test the effect of infection condition (NP and P) within each treatment level (two levels per experiment) and the effect of treatment level within each infection condition. PERMANOVA main tests results are detailed described in a table and pair-wise tests results are represented in figures with lower case letters and in the main text by p values.

The matrix of each experiment containing biomarkers results per treatment and infection condition was normalised and the Euclidean distance calculated. Then, distance among centroids (i.e. the mean position of all the points representing a given sample) was visualized in Principal Coordinates Ordination analysis (PCO). In the PCO graph, the variables (biomarkers) that best explained the samples spatial distribution were represented as superimposed vectors.

## 3. Results

### 3.1. Infection success

Cockles used on each experimental treatment (two levels of salinity, temperature and pH) and infection condition (non-parasitized (NP) and parasitized (P)) of each experiment showed similar mean shell length ($p > 0.05$), i.e. 15.7 mm, ranging between 13 and 17 mm.

For the three experiments, experimentally infected cockles, from thereafter named as “parasitized cockles (P)”, presented significantly higher number of *H. elongata* metacercariae compared to cockles naturally infected, from thereafter named as “non-parasitized (NP) cockles” (Tables 2 and 3). Cockles presented similar infection level when

**Table 2**
Mean (±standard deviation) number of *Himasthla elongata* metacercariae found in dissected cockles for each experiment (salinity, temperature and pH) and each treatment (two levels per experiment). NP: non-parasitized cockles; P: parasitized cockles.

| | Mean ± SD | | | |
|---|---|---|---|---|
| Salinity | 30 | | 22 | |
| | NP | P | NP | P |
| | 0.7 ± 0.9 | 5.6 ± 1.5 | 0.8 ± 1.0 | 7.1 ± 2.1 |
| Temperature | 17 °C | | 22 °C | |
| | NP | P | NP | P |
| | 0.2 ± 0.9 | 6.5 ± 5.3 | 0.7 ± 0.8 | 7.1 ± 2.9 |
| pH | 8.2 | | 7.8 | |
| | NP | P | NP | P |
| | 0.3 ± 0.5 | 4.1 ± 2.0 | 0.6 ± 0.5 | 6.9 ± 2.2 |

exposed to lower salinity (7.1 ± 2.1 metacercariae cockle$^{-1}$) compared to control salinity (5.6 ± 1.5 metacercariae cockle$^{-1}$) (Tables 2 and 3). Likewise, *H. elongata* abundance was similar in cockles infected at 22 °C (7.1 ± 2.9 metacercariae cockle$^{-1}$) compared to cockles infected at control temperature, 17 °C (6.5 ± 5.3 metacercariae cockle$^{-1}$) (Tables 2 and 3). In the pH experiment, *H. elongata* infection was higher when cockles were infected at lower pH (7.8) (6.9 ± 2.2 metacercariae cockle$^{-1}$) compared to control pH (8.2) (4.1 ± 2.0 metacercariae cockle$^{-1}$) (Tables 2 and 3).

### 3.2. Biochemical descriptors

#### 3.2.1. Salinity experiment

Salinity treatment and infection condition as well as the interaction between factors presented no significant effect on PROT and GLY content (Table 4).

Salinity treatment did not affect SOD activity in NP neither P cockles (Table 4). Trematode infection exerted an effect on SOD activity (Table 4) with significantly higher SOD values in NP compared to P cockles within both salinity treatments ($p < 0.05$, Fig. 2A). The interaction between salinity and infection conditions presented no effect on SOD activity (Table 4).

Overall, salinity treatment presented no significant effect on CAT activity (Table 4). However, within P cockles, salinity proved do exert an effect on CAT activity with lower values when cockles were exposed to salinity 22 compared to salinity 30 ($p < 0.05$, Fig. 2B). Trematode infection significantly affected CAT activity (Table 4), especially noticed at the lowest salinity treatment when NP cockles presented significantly higher CAT activity compared to P cockles ($p < 0.01$, Fig. 2B). PERMANOVA revealed a significant effect of the interaction between salinity and infection condition on CAT activity (Table 4).

**Table 3**
Two-way ANOVAs results performed to test the effects of experimental treatments, infection condition and interaction of factors on the number of *Himasthla elongata* metacercariae found in dissected cockles. MS: mean square; F: F value; p: p value. Bold values indicate significant differences ($p < 0.05$).

| | | Two-way ANOVAs | | |
|---|---|---|---|---|
| | | MS | F | p |
| Salinity | Treatment | 0.151 | 0.828 | 0.370 |
| | Infection condition | **68.972** | **377.648** | **<0.001** |
| | Interaction | 0.066 | 0.358 | 0.554 |
| | Error | 0.183 | | |
| Temperature | Treatment | 0.490 | 2.066 | 0.163 |
| | Infection condition | **18.862** | **79.477** | **<0.001** |
| | Interaction | 0.007 | 0.028 | 0.868 |
| | Error | 0.237 | | |
| pH | Treatment | **0.819** | **5.861** | **0.022** |
| | Infection condition | **18.567** | **132.857** | **<0.001** |
| | Interaction | 0.209 | 1.493 | 0.231 |
| | Error | 0.140 | | |

Salinity treatment showed to have no influence on GPx activity (Table 4). Infection condition significantly affected GPx activity (Table 4) within salinity treatment 22, with higher values registered for NP compared to P cockles (Fig. 2C). There was also a significant effect of the interaction of factors (salinity and infection) on the GPx activity (Table 4).

Salinity treatment and infection condition as well as the interaction between factors presented no significant effect on GSTs activity (Table 4).

Salinity treatment presented an effect on ETS levels (Table 4) with a different trend according to infection condition. Within NP cockles, ETS was significantly lower at salinity 30 compared to the lowest salinity treatment ($p < 0.05$, Fig. 2D), while within P cockles ETS was significantly higher at control salinity (30) compared to salinity 22 ($p < 0.01$, Fig. 2D). Infection condition exerted a significant effect on ETS activity (Table 4) with significantly higher activity in P cockles compared to NP cockles within control salinity (30) ($p < 0.01$, Fig. 2D) and significantly lower activity in P cockles compared to NP cockles exposed to low salinity treatment ($p < 0.01$, Fig. 2D). PERMANOVA showed also a significant effect of the interaction between salinity and infection on the ETS activity (Table 4).

Salinity treatment affected LPO levels (Table 4). Nevertheless, this effect was only significant within P cockles ($p < 0.05$, Fig. 2E) with significantly higher LPO levels at salinity 30 compared to low salinity treatment (22). Infection condition presented a significant effect on LPO (Table 4) at low salinity treatment (22), with LPO levels significantly lower in P compared to NP cockles ($p < 0.01$, Fig. 2E). Interaction between salinity and infection showed a significant effect on LPO levels (Table 4).

The PCO horizontal dimension (Axis 1) explained 69% of the total variation separating NP cockles, in the negative side of the axis, from P cockles, in the positive side of the axis. SOD, CAT, GPx and LPO were the variables that better explained this variation presenting high negative correlation with axis 1 ($r > -0.8$). Axis 2 explained 25% of the total variation which separated P cockles exposed to control salinity, in the positive side of the axis, from the other conditions with a strong positive correlation with ETS activity ($r > 0.8$) (Fig. 2F).

#### 3.2.2. Temperature experiment

Temperature treatment, infection condition and interaction between factors presented no significant effect on PROT and GLY content (Table 4).

Temperature treatment showed an effect on SOD activity (Table 4), mainly within NP cockles, with significantly higher levels when cockles were at control temperature (17 °C) compared to cockles from the 22 °C treatment ($p < 0.05$, Fig. 3A). Infection condition presented a significant effect on SOD activity (Table 4) especially noticed when cockles were exposed to the higher temperature treatment (22 °C) and with significantly higher SOD values in NP compared to P cockles ($p < 0.05$, Fig. 3A). Despite lower SOD values recorded in P cockles exposed to 22 °C, no significant effect of the interaction between temperature and infection on SOD activity was observed (Table 4).

Temperature treatment affected CAT activity within P cockles, with significantly lower CAT values when cockles were exposed to the higher temperature compared to control temperature ($p < 0.05$, Fig. 3B). Infection condition presented a significant effect on CAT activity (Table 4) within 22 °C treatment with NP cockles presenting significantly higher CAT than P cockles ($p < 0.05$, Fig. 3B). The interaction between temperature and infection did not affect CAT activity (Table 4).

Temperature presented a significant effect on GPx activity (Table 4) with significantly higher levels at 17 °C compared to 22 °C ($p < 0.05$, Fig. 3C). Infection condition affected the GPx activity (Table 4) within the control temperature treatment with NP cockles presenting significantly higher GPx levels compared to P cockles ($p < 0.01$, Fig. 3C). PERMANOVA demonstrated no significant effect of the interaction between factors on GPx activity (Table 4).

**Table 4**
PERMANOVAs results performed to test the effects of experimental treatments, infection condition and interaction of factors on the biochemical descriptors. PROT: protein; GLY: glycogen; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GSTs: glutathione S-transferases; ETS: electron transport system; LPO: lipid peroxidation; MS: mean square; F: F value; p: p value. Bold values indicate significant differences ($p < 0.05$).

| | | Treatment | | | Infection condition | | | Interaction | | | Error |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MS | Pseudo-F | p | MS | Pseudo-F | p | MS | Pseudo-F | p | MS |
| Salinity | PROT | 0.017 | 0.057 | 0.817 | 7.059 | 23.185 | 0.059 | 1.487 | 4.88 | 0.062 | 0.304 |
| | GLY | 0.580 | 0.727 | 0.420 | 1.299 | 1.627 | 0.233 | 2.731 | 3.420 | 0.105 | 0.799 |
| | SOD | 0.002 | 0.005 | 0.946 | **7.905** | **21.368** | **0.002** | 0.134 | 0.361 | 0.562 | 0.370 |
| | CAT | 0.425 | 1.218 | 0.302 | **3.768** | **10.791** | **0.012** | **4.013** | **11.495** | **0.012** | 0.349 |
| | GPx | 0.007 | 0.034 | 0.857 | **6.071** | **30.878** | **<0.001** | **3.349** | **17.033** | **0.003** | 0.197 |
| | GSTs | 0.973 | 0.986 | 0.347 | 1.082 | 1.098 | 0.330 | 1.056 | 1.071 | 0.325 | 0.986 |
| | ETS | **1.792** | **13.264** | **0.008** | **1.039** | **7.687** | **0.024** | **7.088** | **52.446** | **<0.001** | 0.135 |
| | LPO | **2.292** | **7.389** | **0.037** | **3.994** | **12.877** | **0.008** | **2.233** | **7.202** | **0.029** | 0.310 |
| Temperature | PROT | 2.726 | 8.545 | 0.061 | 3.997 | 12.529 | 0.064 | 1.725 | 5.406 | 0.059 | 0.319 |
| | GLY | 0.331 | 0.305 | 0.602 | $1.10^{-4}$ | $1.10^{-4}$ | 0.992 | 1.988 | 1.832 | 0.211 | 1.085 |
| | SOD | **4.500** | **11.549** | **0.008** | **3.164** | **8.121** | **0.021** | 0.218 | 0.560 | 0.475 | 0.390 |
| | CAT | 0.005 | 0.008 | 0.931 | **3.616** | **6.109** | **0.040** | 2.643 | 4.465 | 0.064 | 0.592 |
| | GPx | **6.662** | **24.368** | **0.001** | **2.150** | **7.864** | **0.028** | $2.10^{-4}$ | $6.10^{-4}$ | 0.980 | 0.273 |
| | GSTs | 1.121 | 1.243 | 0.291 | 2.160 | 2.396 | 0.157 | 0.508 | 0.563 | 0.477 | 0.901 |
| | ETS | **2.455** | **29.942** | **0.002** | **7.036** | **57.159** | **<0.001** | 0.525 | 4.266 | 0.072 | 0.123 |
| | LPO | **6.770** | **35.373** | **<0.001** | **2.122** | **11.086** | **0.010** | 0.578 | 3.019 | 0.121 | 0.191 |
| pH | PROT | 2.006 | 2.433 | 0.162 | 1.574 | 1.909 | 0.202 | 0.824 | 0.999 | 0.343 | 0.824 |
| | GLY | 6.245 | 10.596 | 0.060 | $4.10^{-6}$ | $6.10^{-6}$ | 0.998 | 0.041 | 0.069 | 0.805 | 0.589 |
| | SOD | 1.945 | 5.734 | 0.050 | **6.106** | **18.001** | **0.003** | 0.236 | 0.696 | 0.426 | 0.339 |
| | CAT | 1.639 | 2.735 | 0.135 | 0.097 | 0.162 | 0.702 | **4.471** | **7.461** | **0.024** | 0.599 |
| | GPx | 2.247 | 2.394 | 0.158 | 1.162 | 1.239 | 0.297 | 0.084 | 0.090 | 0.769 | 0.938 |
| | GSTs | 0.295 | 0.338 | 0.575 | 2.448 | 2.802 | 0.135 | 1.270 | 1.454 | 0.267 | 0.873 |
| | ETS | 2.071 | 3.730 | 0.091 | 0.452 | 0.814 | 0.392 | **4.035** | **7.268** | **0.028** | 0.555 |
| | LPO | 1.191 | 1.200 | 0.299 | 0.372 | 0.375 | 0.555 | 1.499 | 1.511 | 0.259 | 0.992 |

Temperature treatment, infection condition and interaction between factors presented no significant effect on GSTs activity (Table 4).

Temperature affected ETS activity (Table 4) noticed within NP cockles with significantly higher values in cockles exposed to temperature control in comparison to cockles exposed to 22 °C treatment ($p < 0.01$, Fig. 3D). Infection condition significantly affected ETS levels (Table 4) with significantly higher activity in P compared to NP cockles ($p < 0.05$, Fig. 3D). PERMANOVA results demonstrated that the interaction between temperature and infection did not affect ETS levels (Table 4).

Temperature presented a significant effect on LPO levels (Table 4) with significantly higher LPO when cockles were exposed to 22 °C compared to cockles from the control temperature treatment ($p < 0.04$, Fig. 3E). Infection condition also affected LPO levels (Table 4) within temperature treatment 22 °C with P cockles presenting significantly higher LPO levels than NP cockles ($p < 0.05$, Fig. 3E). There was no significant effect of the interaction between temperature and infection on LPO levels (Table 4).

The PCO axis 1 explained 63% of the total variation separating the control condition, i.e. NP cockles exposed to 17 °C, in the negative side

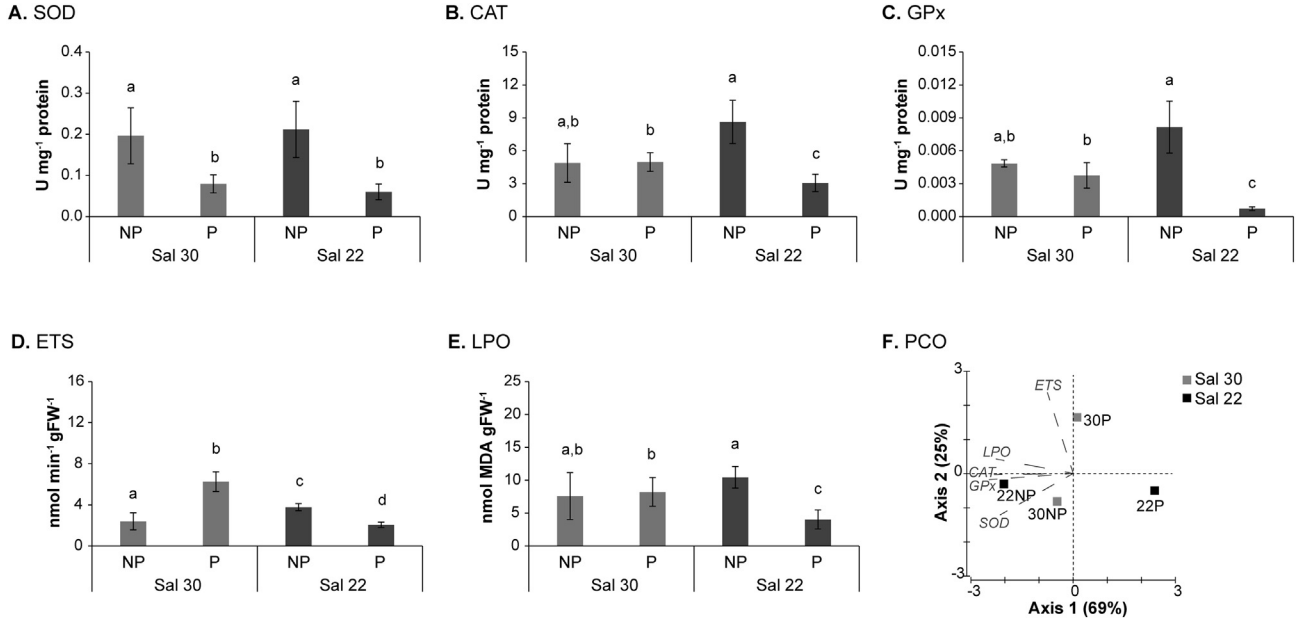


**Fig. 2.** Salinity experiment. Mean values (±standard deviation) and significant differences represented with different lower case letters of A: SOD, superoxide dismutase activity; B: CAT, catalase activity; C: GPx, glutathione peroxidase activity; D: ETS, electron transport system activity and E: LPO, lipid peroxidation levels in non-parasitized (NP) and parasitized (P) cockles with *Himasthla elongata*. F: Principal coordinates ordination analysis (PCO) showing the variables that better explained samples distribution.

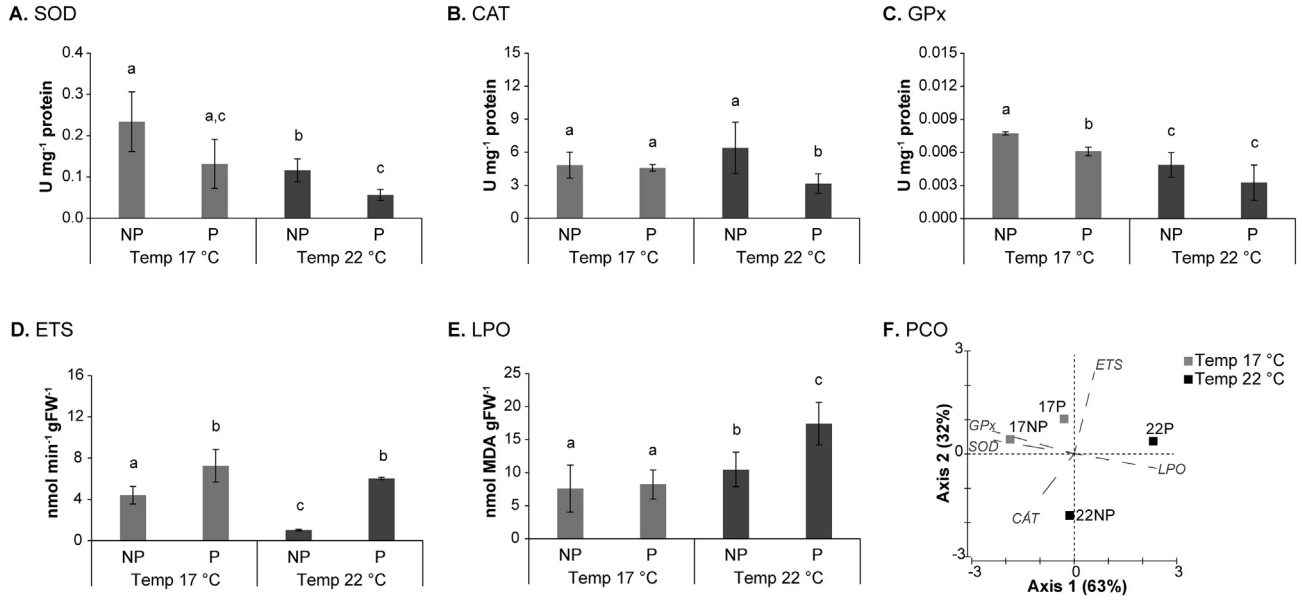


**Fig. 3.** Temperature experiment. Mean values (±standard deviation) and significant differences represented with different lower case letters of A: SOD, superoxide dismutase activity; B: CAT, catalase activity; C: GPx, glutathione peroxidase activity; D: ETS, electron transport system activity and E: LPO, lipid peroxidation levels in non-parasitized (NP) and parasitized (P) cockles with *Himasthla elongata*. F: Principal coordinates ordination analysis (PCO) showing the variables that better explained samples distribution.

of the axis, from P cockles exposed to higher temperature (22 °C), in the positive side of the axis. SOD and GPx presented high negative correlation ($r > -0.8$) and LPO high positive correlation ($r > 0.8$) with axis 1 and together were the variables that better explained the samples separation. Axis 2 explained 32% of the total variation separating NP cockles exposed to higher temperature (22 °C), in the negative side of the axis, from the remaining conditions with a strong positive correlation with ETS activity ($r > 0.8$) and a strong negative correlation with CAT activity ($r > -0.7$) (Fig. 3F).

### 3.2.3. pH experiment

There was no effect of pH, infection neither interaction between those on the PROT and GLY content (Table 4).

Different levels of pH exerted no effect on SOD activity (Table 4). Infection condition presented a significant effect on SOD levels (Table 4) with significantly higher activity in NP compared to P cockles ($p < 0.05$, Fig. 4A). PERMANOVA results demonstrated no significant effect of the interaction on SOD activity (Table 4).

The pH treatment and infection condition did not affect CAT activity (Table 4). However, there was a significant effect of the interaction between pH and infection on CAT activity (Table 4) with significantly lower CAT when cockles were exposed to both lower pH and infection (Fig. 4B).

There was no effect of pH, infection neither interaction between those on the GPx and GSTs activity (Table 4).

The pH treatment and infection condition exerted no effect on ETS activity (Table 4), except for NP cockles between both pH levels

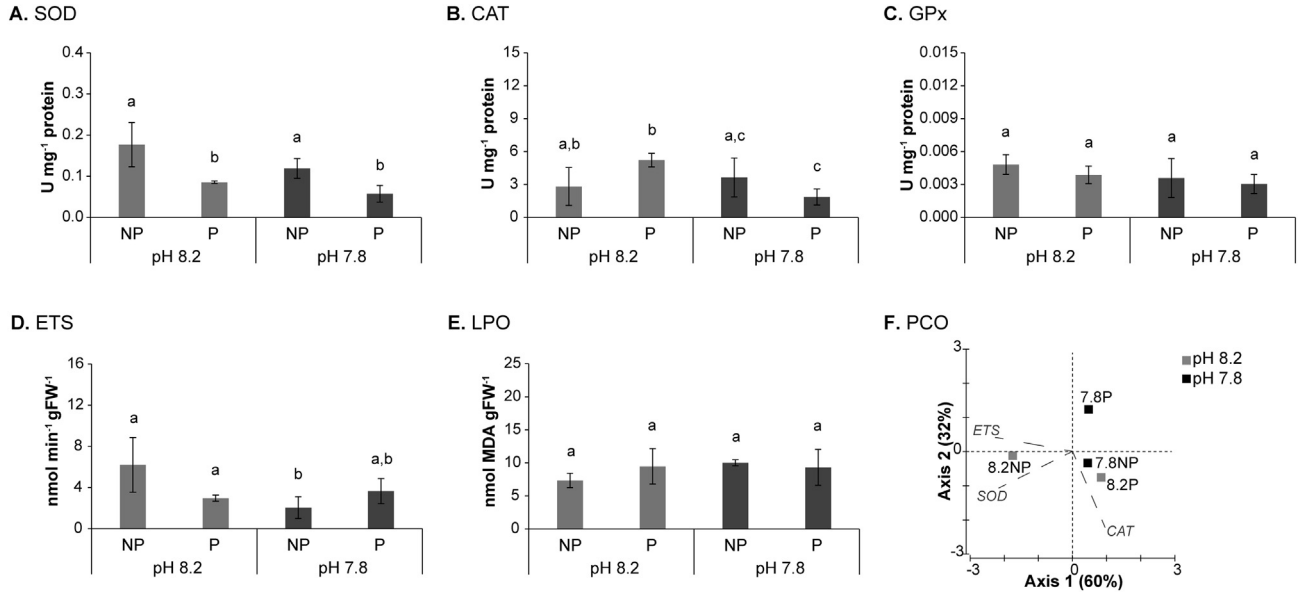


**Fig. 4.** pH experiment. Mean values (±standard deviation) and significant differences represented with different lower case letters of A: SOD, superoxide dismutase activity; B: CAT, catalase activity; C: GPx, glutathione peroxidase activity; D: ETS, electron transport system activity and E: LPO, lipid peroxidation levels in non-parasitized (NP) and parasitized (P) cockles with *Himasthla elongata*. F: Principal coordinates ordination analysis (PCO) showing the variables that better explained samples distribution.

(Fig. 4D). PERMANOVA revealed a significant effect of the interaction of factors (Table 4).

There was no effect of pH, infection neither interaction between those on the LPO levels (Table 4).

The PCO axis 1 explained 60% of the total variation separating the control condition, i.e. NP cockles exposed to pH 8.2, in the negative side of the axis, from the other three conditions, in the positive side of the axis. SOD and ETS activities were the variables that better explained the variation, presenting high negative correlation ($r > -0.8$) with axis 1. The PCO vertical dimension (Axis 2) explained 32% of the total variation separating P cockles exposed to lower pH (7.8), in the positive side of the axis, from the other conditions with a strong negative correlation with CAT activity ($r > -0.8$) (Fig. 4F).

## 4. Discussion

Parasites are strongly influenced by environmental conditions and rely on their hosts for completion of the life cycle. A change in these factors, particularly salinity, temperature and pH, are very likely to differentially affect the parasite, the host and/or their interaction. The outcome of such changes on host–parasite relationships in marine systems has rarely been assessed, the present study being one of the first that experimentally investigated the impact of predicted climate change on trematode parasitism by testing different levels of salinity, temperature and pH on an intertidal trematode–bivalve system.

It is generally admitted that older and consequently larger cockles are more heavily infected due to a longer exposure period to parasites, and also because larger cockles present higher filtration rate which is the main way of infection (Wegeberg et al., 1999; Mouritsen et al., 2003). On the other hand, it was also demonstrated that cockles from a particular shell length range could be more vulnerable to infection (de Montaudouin et al., 2012). For these reasons, it is important to mention that the mean shell length of the cockles used in the experiments were all similar (mean = 15.7 mm). Moreover, and by choosing small size cockles, we obtained cockles with very low natural infection (mean infection ranged between 0.2 and 0.8 metacercariae per cockle).

### 4.1. Salinity effects in cockles trematode infection

The present study showed that, although without statistical significance, cockles from the lowest salinity treatment presented higher number of *H. elongata* metacercariae than cockles from the control salinity. There are few studies reporting effects of salinity on marine trematode parasites and the majority of them are related to cercariae emergence from its snail first intermediate host (Mouritsen, 2002; Lei and Poulin, 2011; Studer and Poulin, 2012). Lei and Poulin (2011) and Studer and Poulin (2012) showed that cercariae emergence from first intermediate host increased with increasing salinity, while Mouritsen (2002) demonstrated the same trend but only under elevated temperatures. In contrast, Koprivnikar and Poulin (2009) and Koprivnikar et al. (2014) showed cercariae emergence increase under decreasing salinities. Considering that Studer and Poulin (2012) studies, on a snail (1st host) - trematode - amphipod (2nd host) system, showed the highest cercariae survival at salinities ranging between 30 and 40 leading to greater successful transmission, our results demonstrate that cockles infection: i) was independent on the different cercariae performance according to salinity; ii) but was affected by the performance of cockles at lower salinities. Previous studies developed by Malham et al. (2012) demonstrated that *C. edule*, considered as an euryhaline species, i.e. an organism able to adapt to a wide range of salinities, shows its optimal salinity between 30 and 35 (Malham et al., 2012). Salinities outside this range could be stressful to cockles, in particular lower salinities because it might be expected that intertidal mollusks would more routinely experience elevated rather than decreased salinities, particularly during sunny low tides (Koprivnikar and Poulin, 2009). Therefore, higher number of cercariae in cockles exposed to lower salinity may result from cockles abnormal performance under this condition, being more susceptible to infection. In fact, non-parasitized cockles from the low salinity treatment showed higher antioxidant activity than non-parasitized cockles at control salinity, evidencing the negative impacts induced to cockles by low salinity. Similar harmful effects of low salinities were observed in cockles (Gonçalves et al., 2017), in *Mytilus galloprovincialis* mussels (Freitas et al., 2017) and in *Ruditapes philippinarum* clams (Velez et al., 2016) from the same coastal lagoon. Nevertheless, the observed response was different when cockles were parasitized. In general, when a trematode invades a bivalve, its immune system answers by recruiting granulocytes (a type of haemocytes). Granulocytes have dense cytoplasmic granules and are known to be the main immunoreactive cells in the bivalve immune system, presenting the highest phagocytic capacity, destroying the invader with lysosomal enzymes and producing more ROS (Lin et al., 2013; Soudant et al., 2013). However, under both salinities tested, parasitized cockles seemed to be subjected to lower oxidative stress than non-parasitized cockles, since antioxidant defences and cellular damages were lower in infected cockles. This response was also accompanied by a reduction in the metabolic activity (lower ETS) when cockles were parasitized and exposed to low salinity. The apparent less stressful condition, in parasitized cockles, could instead indicate that cockles are experiencing an enzymatic inhibition by any mechanism triggered by parasites such as a reduction in some or all functions of the immune system described above. Moreover, cockles experiencing two stressful conditions at the same time, low salinity and trematode infection, seem to react with a metabolic reduction emphasizing the significant capacity of trematodes in modifying the hosts response to a given stress (e.g. Macleod and Poulin, 2016). Low metabolic capacity measured by the activity of the mitochondria respiratory chain (ETS) resulted into low ROS generation which may prevent cellular damages, explaining low LPO levels in parasitized cockles at low salinity. *R. philippinarum* showed a similar response when exposed to both low salinity and *Vibrio tapetis* by significantly reducing the lysozymes level, a possible sign of overall metabolism reduction, although in this case the strategy of defence adopted led to a higher disease prevalence and progression (Reid et al., 2003).

### 4.2. Temperature effects in cockles trematode infection

It is well stablished that under increasing temperatures there is an increase on cercariae activity leading to a faster depletion of their finite energy reserves which in turn reduces their survival rates (Studer et al., 2010; Studer and Poulin, 2013). However, due to this short-term increasing activity, with warmer temperatures, the number of interactions between cercariae and hosts per unit of time is higher (Evans, 1985) which may increase the effective infectivity (Thieltges and Rick, 2006; Studer et al., 2010). In one hand, these arguments can explain the slightly higher number of *H. elongata* metacercariae infecting cockles at 22 °C compared to cockles maintained at 17 °C, but on the other hand, the difference was not statistically different maybe because both temperatures are in the range of the optimal infection temperature (between 15 °C and 22 °C) described for other Himasthlidae parasites (de Montaudouin et al., 2016). Biomarkers showed that temperature affected cockles enzyme activity, with high temperature treatment inhibiting the antioxidant response in both infection conditions. Under temperature stressful conditions (22 °C) and compared to control (17 °C), a strong reduction in non-parasitized cockles metabolic capacity was observed, which may explain that cockles were not able to activate their antioxidant defence system, leading to increased LPO levels as previously described for *R. decussatus* (Velez et al., 2017). Despite an overall increase in the metabolic activity in parasitized cockles compared to non-parasitized cockles regardless temperature treatment, when cockles were facing two stressful conditions at the same time (trematode infection and high temperature) an inhibition of their antioxidant capacity was observed. This increase of the metabolic rate (measured

through ETS activity) in parasitized cockles exposed to temperature 22 °C generated higher ROS levels, which in turn resulted in the higher production of ROS that, associated to lower antioxidant capacity lead to higher LPO levels in parasitized cockles. Generally, infected cockles have to face an additional metabolic requirement in order to supply parasites with sufficient energy to grow (MacLeod, 2017). Overall, the present results demonstrated that higher temperature may cause an additional stress to parasitized cockles, inducing higher metabolic activity and cellular damages.

### 4.3. Effects of water acidification in cockles trematode infection

Research on the ecological impacts of ocean acidification has expanded (Poulin et al., 2016) but, the interactive effects of parasitism and ocean acidification on marine organisms is still rudimentary. Nevertheless, there are already some evidences of cercariae longevity reduction (MacLeod and Poulin, 2015), although with higher trematode infection success, under low pH conditions (Harland et al., 2015; Harland et al., 2016). In comparison to the most studied bivalve species (e.g. *Mytilus edulis*), *C. edule* has a thinner periostracum (Richardson et al., 1981) and its shell is only composed by aragonite, the most soluble polymorph of carbonate (Cubillas et al., 2005). Therefore, cockles are particularly vulnerable to acidification which decreases their body condition (Schade et al., 2016) likely inducing a shift on their immune system and increasing the cockles susceptibility to diseases. Accordingly, in the present study, cockles from the lower pH treatment presented significantly higher number of *H. elongata* metacercariae, possibly related to higher host susceptibility mostly evidenced by the antioxidant system inhibition, lower metabolism and higher cellular damages experienced by cockles exposed to these conditions. Biomarkers showed that pH affected non-parasitized cockles enzyme activity, with low pH treatment inhibiting the antioxidant response. Under pH stressful conditions (7.8), a strong reduction in non-parasitized cockles metabolic capacity was observed as previously demonstrated for *M. galloprovincialis* mussels (Freitas et al., 2017). Under both pH treatments, but especially noticed at 7.8, parasitized cockles tended to decrease their enzymatic activity in comparison to non-parasitized cockles. Previous studies already demonstrated that higher $pCO_2$ levels impact bivalves immune system reducing the bactericidal activity (Ellis et al., 2015) and the phagocytic activity by decreasing the lysosomes health (Bibby et al., 2008). This mechanism, similarly to what was described for low salinity effects, reduces overall ROS production preventing the occurrence of LPO and limiting the activation of antioxidant enzymes.

Considering the three experiments and taking into account non-parasitized cockles, salinity was the factor exerting the highest stress levels on cockles. This was demonstrated by higher antioxidant response resulting into greater injuries to cockles when exposed to lower salinity condition. Considering parasitized cockles, our findings may indicate that the interaction trematode infection × temperature and trematode infection × pH will result into major shifts in the cockles biochemical performance, with greater impacts at high temperature and low pH leading to possible higher disease susceptibility.

## 5. Conclusions

The present work showed that the integration of parasitology into the physiological assessment of marine organisms exposed to simulated climate change conditions is urgently required. A failure to do so may lead to the incorrect identification of some marine species as tolerant or susceptible to a given stress, when in fact the physiological response of the organism is modified by parasitic infection.

Our results suggest that changes forecasted by many models may promote the proliferation of the parasites infective stages in many ecosystems leading to enhanced transmission, especially on temperate regions, that will influence the geographical distribution of some diseases and, probably, the survival capacity of infected bivalves. As parasites have the ability to influence host population behaviour, reproduction and survival, small changes will have a multitude of potential subsequent effects on host populations and communities. An increase in overall parasite abundance could alter marine ecosystems in significant ways: populations of first intermediate host snail species, usually castrated by trematode infection (Lafferty and Kuris, 2009), would have lower reproductive potential; second intermediate hosts parasite-mediated dynamics, such as predator-prey relationships (Thomas et al., 1998) or bottom-up control (Johnson and Heard, 2017) would be maximised; and any host species that experience increased mortality as a consequence of parasitic infection (Desclaux et al., 2004; Thieltges, 2006) may become rare.

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